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Genetic dissection of *Plasmopara viticola* population from a Greek vineyard in two consecutive years

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Abstract

Oilspots of the *Plasmopara viticola* population in a Greek vineyard over 2 years were collected and the pathogen genotyped with the use of four microsatellite loci. In 2001, five samplings occurred and 327 lesions were collected, which were classified to 23 genotypes. In 2002, the 426 lesions collected in four samplings belonged to 54 genotypes. A reproducible pattern of the evolution of the epidemic was exhibited that can be described as 'predominance-of-one-clone'. In 2001, the predominant clone covered 72–92% of each sample, while in 2002 the incidence of the predominant clone was 38–90%. The remaining genotypes showed low clonal reproduction and dispersal. Oosporic infections occurred throughout the season. The severity and the diversity in 2002 were, however, higher than in 2001 and this was not in accordance with the climatic conditions, suggesting that other factors, like the epidemic of the previous year, also influence the epidemic of the disease. These results produce new concepts about the epidemiology of the pathogen.

Introduction

The Oomycete *Plasmopara viticola*, the causal agent of grapevine downy mildew disease, was introduced to Europe (France) in 1878 from North America through the resistant-to-phylloxera wild American vine rootstocks. In Greece, its presence was first observed in 1881 in the Messinia region of Peloponnesus (Gennadios, 1889) and, within 10 years, was spread to the whole Peloponnesus, Ionian Islands and around Athens, in Attica. The first catastrophic epidemic occurred in 1900, when, in a short time, two-thirds of the expected yield were destroyed (Sarejanni, 1951). Since then, the disease has been a permanent threat in viticultural areas of the main land of Greece and in the Ionian Islands.

The life cycle of the pathogen consists of one sexual cycle in autumn and many asexual cycles during the growing season of the grapevine. The sexual stage includes the mating of the gametes that produces the oospore: the overwintering organ of the organism in dead leaf tissues, which may survive for five or more years in the soil (Hill G., SLVA Oppenheim, Germany, pers. comm.). In spring, due to rain precipitation, the oospore germinates to form a macrosporangium, which releases zoospores that cause the primary infections on the young berries or on the leaf surface (in the latter case with the form of 'oily' lesions). The primary lesions give birth to wind-dispersed sporangia that under the presence of leaf wetness cause the secondary infections. Depending on the environmental conditions, numerous clonal cycles may occur in one season leading to abrupt increase in disease severity with a disastrous impact on the yield. The organism is diploid in both sexual and asexual stages.

Up to now, two basic assumptions about the disease epidemiology existed: (1) primary infections last for a limited, less-than-one-month period, and play a role

only at the start of the epidemic, and (2) the secondary cycles of the disease are responsible for the growth of the epidemic because of the high number of secondary infections they produce and the migration of sporangia in a short time to long distances. Based on these assumptions, epidemic development predictions, control strategies design and modeling programs were formed.

In addition to the general concepts mentioned above, under the specific conditions in Greek vineyards, particular epidemiological traits are assumed. Because of the absence of long rains in springtime, which cover the soil with water for many days, oospore germination does not occur on a wide scale but only in specific sites (Zahos, 1959). Usually, the first primary infections occur on 'crawling-on-the-earth' branches of vines near ditches with permanent humidity, on vines in irrigated vineyards, on abandoned, unpruned vines covered with natural vegetation around the vineyard or in abandoned vineyards. Thus, a 'crawling' stage of the disease is suggested before the pathogen 'climbs' on the vine. The rains of May and June trigger the secondary spread of the disease. Later (middle of July up to August) the rise in the temperature in combination with drought halts the epidemic. Regularly, a second round of secondary cycles takes place in autumn, after the first September rainfalls. Oogamy occurs only in autumn, after the temperature decreases to 12 °C.

The existing knowledge, has failed to provide effective control strategies. Successful control has always been a random event, depending more on the quantity of the treatments and the quality of the anti-oomycete substances used. We attribute this failure, on one hand, to the fact that two main assumptions which remain unconfirmed: (1) the qualitative and quantitative contribution of the primary infections to the genetic diversity and epidemiology of the disease is low and (2) the possible distance of sporangia dispersal is great. On the other hand, the role of genotypic diversity of populations as a survival mechanism was disregarded. If conditions that can be lethal for a single individual occur, this does not imply that the population will be eliminated. Consequently, the target-organism of a control strategy should be the whole population instead of an individual. In order to gain this missing knowledge, we decided to study the population genetics of the pathogen, believing that this information can provide us with important knowledge about the biology of the pathogen and the host–pathogen environment.

Materials and methods

Samples collection

All the samplings took place in a 100-vine plot (5 rows × 20 vines, distance between the vines on the rows = 1 m, distance between the rows = 1.5 m), which is placed at the corner of a 1-ha vineyard in the viticultural area of N. Aghialos region (Thessaly domain, Central Greece). The plot is about 0.5 km away from the sea, and the area is mostly covered with vineyards and olive tree orchards. The cultivar of the grapevine is the *Vitis vinifera* Roditis, a local, traditional Greek wine variety, planted in a Royal training system. The plot was not treated against downy mildew during the 2 years of sampling, while the rest of the vineyard was treated. The rain events at that area are random and short, and under these conditions, the secondary cycles of the disease are not numerous and are separated from each other, facilitating the collection of all oilspots and the observation of the spread of the disease.

The date of the first sampling was decided according to the expected time of oospore germination. Normally, the middle of May is the time when downy mildew appears in Greece. At that time, a few days after a rain event (depending on the incubation time, given the average temperature of this period), we searched the plot for primary lesions and collected all the lesions found. Only a part (about one-third) of each lesion was cut and conserved in a 4 °C cool-box. The rest of the lesion was left on the leaf and, as later observed, stayed alive and sporulated. The dates of the next samplings depended on the rain events during the grape-growing period. Every time there was a rain that raised the relative humidity (RH) during the night to up to 100%, we estimated the incubation time according to the temperature (Goidanich, 1982) and we sampled just after the expected day of symptom appearance. Again, all the lesions detected were collected.

Following the previous principles, five samples were collected in 2001 and four in 2002, named 2001a–e and 2002a–d, respectively. The dates of the samplings and the sample sizes are given in Table 1. After 3/07, and until the beginning of August, in 2001, there was almost little precipitation (3.2 mm), the average temperature (Tav) increased to 26 °C (Tav_{max} = 31.5 °C, Tav_{min} = 20 °C) and the average relative humidity (RHav) decreased to 63% ($RHav_{max}$ = 84%, $RHav_{min}$ = 44%). In 2002, the corresponding conditions were 18.2 mm rain, Tav_{max} = 31.5 °C,

Table 1. Lesions collected and analyzed, number of *P. viticola* genotypes determined, number of lesions that belong to the predominant genotypes, new genotypes in each sampling, vines infested by the predominant genotypes, total number of infested vines, genotypic diversity of the nine samples collected in 2001 and 2002 grapevine growing seasons in a Greek vineyard and precipitation during that seasons

Sample	Date of sampling	Rain events (mm)	Lesions collected	Lesions genetically determined	Lesions of predominant genotype	No genotypes	New genotypes	Vines infested		E_H
								By the predominant	By all genotypes	
		6–10/05 : 25.2 13–14/05 : 4.4								
2001a	28/05	26–27/05 : 10.8	51	47	43 (92%)	5	5	11	15	0.11
2001b	5/06		56	50	41 (82%)	7	5 (71%)	23	30	0.2
2001c	15/06	7/06 : 3.6	94	83	65 (78%)	12	5 (42%)	40	52	0.23
2001d	27/06	20/06 : 0.2	64	61	49 (80%)	9	4 (44%)	45	60	0.21
2001e	10/07	2/07 : 16	62	61	44 (72%)	12	5 (41%)	49	65	0.3
Total population	60.2 mm	327	302	242	23				0.18	
2002a	16/05	8–13/05 : 7.8	2	2		2	2	—	2	
2002b	23/05		54	48	43 (90%)	5	4 (80%)	8	14	0.12
2002c	13/06	22–26/05 : 1.6	152	143	83 (58%)	22	18 (82%)	57	93	0.35
2002d	2/07	25/06 : 3.2	217	137	52 (38%)	41	30 (73%)	65	95	0.53
Total population	12.6 mm	426	330	178	54				0.37	

$Tav_{min} = 20.6\,^{\circ}C$, $RHav_{max} = 81\%$ and $RHav_{min} = 43.7\%$. Under these conditions, production of conidiophores, sporulation and infection were not possible and the disease was not dispersed.

Microsatellite locus amplification and genotyping

The oilspots collected were lyophilized and the plant and fungal DNA were extracted. The DNA was used for PCR amplification of four microsatellite loci using fluorescence-dyed primers specific to the pathogen. The markers BER and ISA consisted in $(TC)_n$ repeats, the marker CES in $(TC)_n(AC)_n$ repeats and the marker GOB in $(CT)_n(CTAT)_n$ repeats. The products of the reactions were visualized by sequence-assisted fragment analysis, and the eight alleles of each isolate were identified. The procedure is described in Gobbin et al. (2003a). The classification in genotypes followed the subsequent principles: lesions presenting the same allele pattern were considered as clones of the same oospore, while the ones presenting a different allele pattern were considered to derive from different oospores.

Genetic analysis and spatial distribution

Gene diversity was studied separately for each sample using clone-corrected data. For each locus, the number of alleles, allele frequency and gene diversity (Nei, 1973) were calculated using Fstat software (Goudet, 1995 – version 2.9.3.1). The allele frequencies were calculated using both clone-corrected and without clone-correction data. The same software was used for the population differentiation based on allele frequencies differences.

Genotypic diversity was estimated using individual genotypes, generated from the four microsatellite loci. For that reason, the Shannon's index (H), which accounts for both abundance and evenness of the genotypes distribution, was calculated (Krebs, 1989) according to the formula

$$H = -\sum_{j=1}^{S} p_i \ln p_i$$

where p_i is the frequency of isolates with the ith genotype and S is the number of different genotypes in the sample. Shannon's equitability (E_H) was then calculated by dividing H by H_{max} (here $H_{max} = \ln N$, N = number of individuals in the sample). Equitability assumes a value between 0 and 1 with 1 being complete evenness.

$$E_H = H/H_{max} = H/\ln N$$

Spatial distribution of the predominant genotypes was analyzed by calculating the distances of the clones from the vine where the genotype first appeared. For each sample, the distribution of the distances was represented with the help of the SYSTAT (version 10) and the median of the distributions were calculated (in Microsoft Excel), in order to describe the spread of the genotypes.

Departures from the Hardy–Weinberg equilibrium (HWE) were tested for each sample (except of the 2002a because it consisted of only two lesions) by the Markov chain randomization method of Guo and Thompson (1992) with the use of GENEPOP version 1.2 (Raymond and Rousset, 1995a,b) by setting 1000 dememorization steps and 2000 batches with 1000 iterations each. Locus BER, that possessed three alleles, was tested for HWE by the complete enumeration method, as described by Louis and Dempster (1987).

Results

During the nine samplings, all the lesions detected were collected and analyzed. Missing data, however, could not be avoided, basically, because some lesions were partially necrotic and the quantity of the DNA extracted was not enough for the PCR amplification. The final percentage of successfully analyzed lesions was 92% for 2001 and 78% for 2002 samples. We underline that the molecular test failure was random and that none of the detected lesions was excluded from the collection.

The assigning of a particular lesion to the same or different oosporic infection event as any of the other lesions based on equal or respectively different microsatellite allele pattern was supported by the following data. Given the number of alleles that appeared in the 2001 population (25 GOB alleles, 4 ISA, 7 CES and 2 BER), the possible genotypes to be identified by these four markers are 273×10^3 (number of allele combinations per locus $= n(n+1)/2$, where n = number of alleles). The actual number of 2002 population was only 54 genotypes. In parallel to the very limited amount of different genotypes in the population, the estimated appearance probability of a possible genotype that contains the most frequent alleles at each locus is 0.014. These facts support the hypothesis that the probability of two lesions having the same genotype

being products of independent recombination events is very low and confirm our genotyping principles.

Samples of 2001

In 2001 five samplings took place. Each sample consisted of between 51 and 64 oilspots (Table 1), except for the third sample that was much larger (94 oilspots). The genotypic diversity was constantly low (E_H = 0.11–0.3). One genotype (predominant-2001) was dominating all samples covering 72–92% of each. At the first sampling, the predominant-2001 was localized mainly on 10 vines at the end of the two right rows (Figure 1A) of the plot and the median of its spread was 1 m from the focal vine 97 (vine 17 in last row) (Figure 2A). At the second sampling it reached a maximum distance of 15.6 m from the focus (maximum distance from the focus inside this plot = 17.1 m) and the median of its spread increased to 3.2 m. At the following samples, the new clones of the predominant-2001 appeared about in the same area, increasing the number of vines they infested until they had infested almost half of the plot (49 vines) at the fifth sampling. The rest of the genotypes in each sample either appeared only once or showed very limited asexual reproduction and caused secondary infections to the same vine where they first appeared or to the neighboring plants. The climatic conditions after the 10/07 (no precipitation, Tav = 26.3 °C, Tav_{max} = 31.6 °C, Tav_{min} = 20 °C) halted the disease spread.

Over the entire one-and-a-half-month period of this naturally spread and chemically uninterrupted downy mildew epidemic, 65% of the vines had been infested, but only two vines heavily, by 23 different genotypes. The genotypic diversity of the whole population had been very low (E_H = 0.18). The predominant-2001 genotype had reproduced asexually, 80% of the total 302 lesions collected. Two genotypes had covered 4% of the total population each, 10 genotypes had caused 2–3 lesions and the remaining 10 had caused only one lesion. Although the predominant-2001 had spread all over the plot, the secondary infections of the other genotypes had been localized around the point of the first appearance of the corresponding genotype. Fourteen of these genotypes had been found in one vine, five genotypes in two vines and only three had spread to three vines (Table 2).

Important questions arose as a result of the 2001 epidemic that demanded the 2002 study. (1) Will the starting sites and the focal point of the disease (vines 97, 98) be the same because of a favorable microclimate? (2) Was the 'predominance-of-one-clone' pattern of epidemic a random event, or is it reproducible? (3) Will the predominant genotype have the same ability for sexual reproduction as it had for asexual reproduction? We expected that the analysis of the 2002 samples could solve these problems.

Samples of 2002

In 2002, four samples were collected (Table 1). The disease appeared with two lesions in the middle of the plot (Figure 1B), both placed at the fork of the two main branches of the vines. One week later, the sample was increased to 54 lesions, but the genotypic diversity was very low (E_H = 0.12), since 90% of the lesions belonged to one genotype (predominant-2002). The lesions of the predominant-2002 were localized over eight neighboring vines, focused on vine 48 (vine 8 in middle row). At the third sampling, however, the disease was evenly dispersed over the entire plot and the genotypic diversity increased, as 18 new genotypes had appeared. The predominant-2002 was further spread to 57 vines in total (median of its spread from the focus = 5 m) (Figure 2B) and reached a maximum distance of 12.4 m (that is the maximum distance from the focal vine 48 inside this plot), but its frequency in the sample was reduced to 58%. Apart from the predominant-2002, three other genotypes (genotypes a–c in Figure 1) reproduced clonally to a big number of clones covering 6–9% of the sample (genotype a already existed in the second sample). The remaining genotypes either appeared once or showed only 2–4 oilspots. The increase of the genotypic diversity and the decrease of the presence of the predominant-2002 characterized the last sample as well.

Over the whole period of 1.5 months disease epidemic, 54 different genotypes had been detected inside the vineyard, having infested a total of 95 vines. The diversity of the whole population had been medium (E_H = 0.37). One genotype had reproduced clonally and had spread to the whole plot, having covered 54% of the whole population. Most of the remaining genotypes had showed slow and limited reproduction; four genotypes had covered 1.5–2.5% of the total population, 11 genotypes had produced 2–3 lesions and these 15 genotypes altogether were responsible for the 35% of the total epidemic. The remaining 35 genotypes had appeared only once having caused 9% of the total infections. There had been, however, in contrast to the

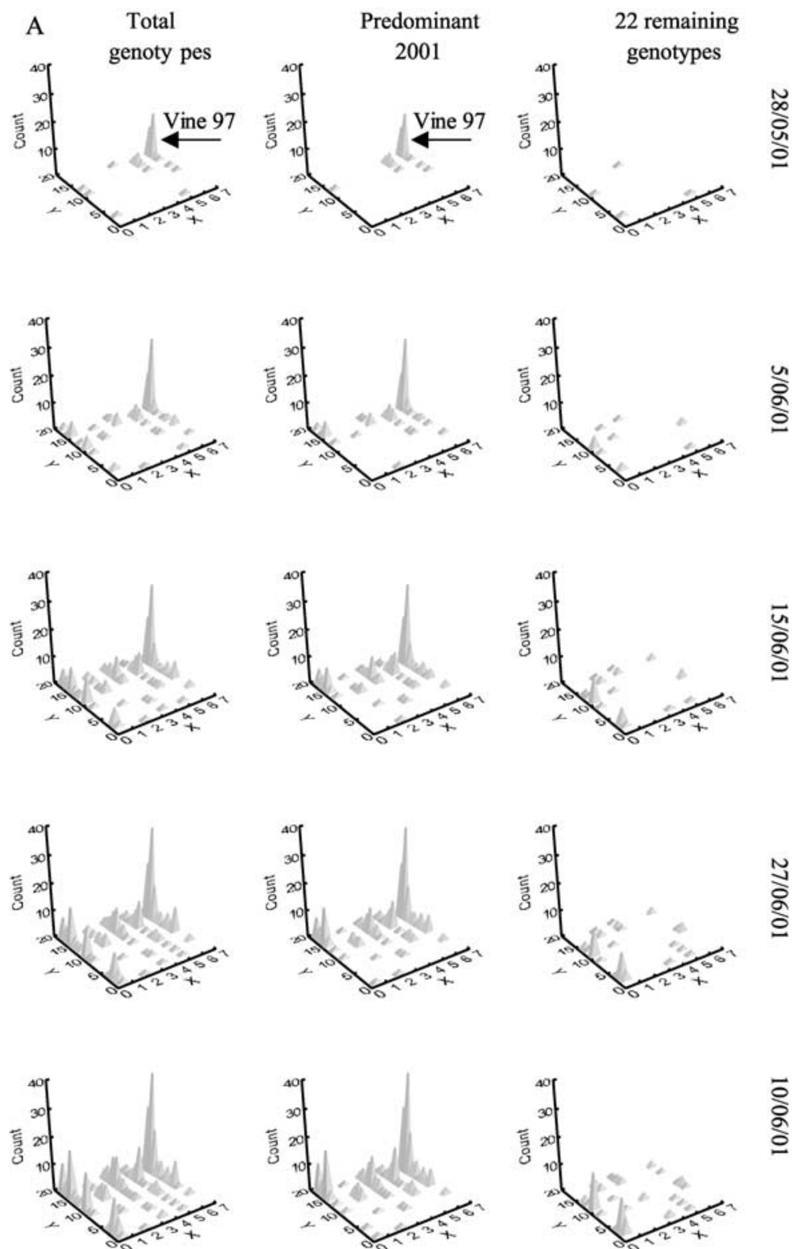

Figure 1. Genetic and spatial dissection of *P. viticola* epidemic in 2001 (A) and 2002 (B) in an untreated vineyard of 100 vines; total genotypes, predominant genotypes, the most widely spread and the remaining genotypes are shown (x = number of rows, y = number of columns, count = number of oilspots of the corresponding genotype(s) cumulative in each sample).

population of 2001, three genotypes that had multiplied up to 8% of the whole population and eight genotypes that had spread to more than three vines (Table 2). The incidence of the disease had been 95% , the infections had been evenly distributed to 94 vines and only one vine (vine 48, the focus of the predominant clone) had been severely attacked. The severity had been much higher than in 2001 – 100 more infections had occurred.

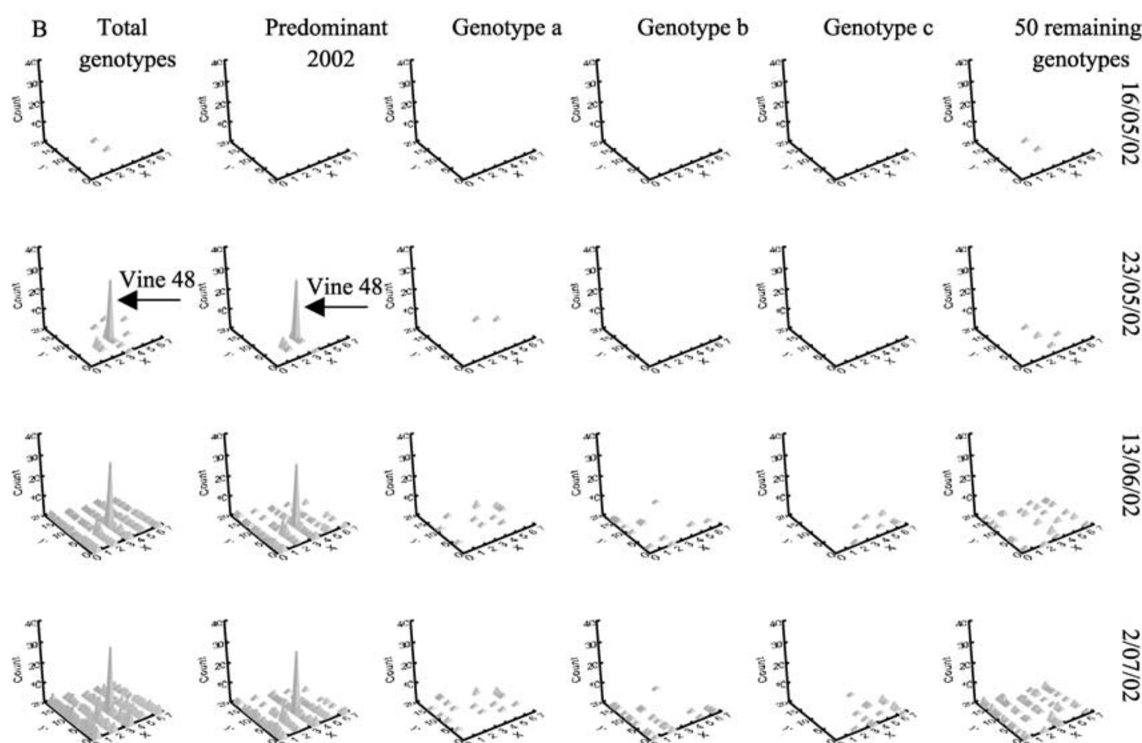

Figure 1. (*Continued*)

Gene diversity and population differentiation

Seven of the eight samples (2002a sample was not included because it consisted of only two lesions) that were tested for HWE were in accordance with the HW expectations at all loci. At the sample 2002d, however, the null hypothesis of random recombination in the population was rejected for locus GOB, but still, over all loci the sample was in equilibrium. Then, the test for population differentiation was performed pair wise between the eight samples, after they were clone-corrected, based on the allele frequencies using the Fstat software. This test demonstrated that the allele frequencies did not differ significantly among the samples, which means that all the samples analyzed along the 2 years come from the one population. In addition to that test, we looked for the same genotypes among the 2 years. It was found that the samples of 2001 do not share any genotypes with 2002 samples. This is in accordance with the inability of the secondary structures of the oomycete (sporangia, mycelium on leaves and branches) to overwinter.

Two of the four microsatellite loci used, GOB and CES, presented very high heterozygosity, locus ISA showed medium and locus BER very low (Table 3). Locus GOB showed high allele diversity (7–32 alleles/sample and 41 alleles in total), CES showed 12 alleles, ISA had only four alleles – allele ISA-144 was very frequent – and BER had two alleles in 2001 and three in 2002, but allele BER-181 was predominant in both years (Figure 3). The populations of 2001 and 2002 as a whole share all their ISA alleles, 20 GOB alleles (49% of the total number of alleles that appeared), 5 CES alleles (42% of the total) and 2 BER alleles. The population of 2001 had five private GOB alleles, while the 2002 population had 16 private alleles, eight of which belonged to genotypes that appeared only once.

Discussion

The genotyping of the individuals of *P. viticola* population in 2001 revealed a population with very low diversity, where one genotype dominated. This genotype

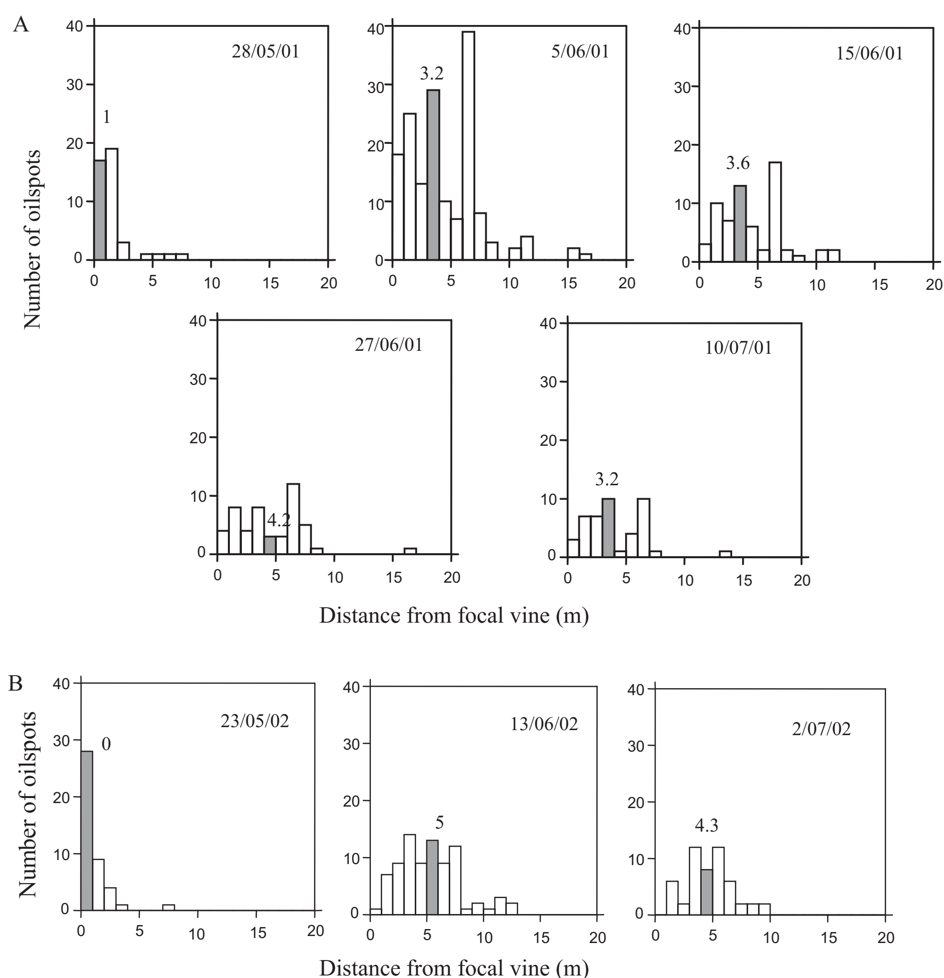

Figure 2. Distance distribution of the oilspots caused by the predominant genotype from the focal vine; (A) predominant-2001 from the vine 97 at five sampling dates, (B) predominant-2002 from the vine 48 at three sampling dates (the median values are also shown on the graph).

Table 2. Characteristics of the *P. viticola* epidemics in an untreated Greek vineyard of 100 vines in 2001 and 2002

Year	Uninfected vines	Vines infected with			Number of genotypes and number of vines they infested**					
		1–9 lesions	10–20 lesions	>20 lesions	1 vine	2 vines	3 vines	4 vines	5 vines	8–23 vines
2001	35	55	8	2 (25, 38)*	14	5	3	—	—	—
2002	5	91	3	1 (31)	35	8	2	2	2	4

*Number of lesions collected on the most severely infected vines.
**The predominant genotypes are excluded.

appeared first with a low number of clones mainly focused on one vine inside the vineyard (and not coming from outside, as it was believed) and then spread to half of the vines. After a repetition of the study in 2002, we observed that the first primary infections appeared in different sites than the previous year, showing that they do not depend on the microclimatic differences inside the 100-vine plot. The first oosporic infections

Table 3. Number of alleles and gene diversity (Nei, 1973) per microsatellite locus and population of *P. viticola*

Locus name	Sample*										
	2001a	2001b	2001c	2001d	2001e	2002b	2002c	2002d	Total 2001	Total 2002	Total (both years)
GOB	7 0.925	11 0.929	17 0.951	12 0.951	14 0.939	9 0.983	24 0.969	32 0.955	25 0.957	36 0.962	41
ISA	2 0.5	4 0.607	4 0.534	2 0.417	4 0.572	3 0.625	3 0.485	4 0.392	4 0.496	4 0.424	4
CES	4 0.7	4 0.762	6 0.803	4 0.795	7 0.837	6 0.867	7 0.805	10 0.816	7 0.798	10 0.816	12
BER	2 0.35	2 0.167	2 0.227	2 0.375	2 0.402	1 0	2 0.338	3 0.185	2 0.361	3 0.249	3

*The sample 2002a is not included because it is constituted of only two lesions.

occurred in the middle of the vineyard and one of these initial genotypes dominated the population. The focus of the predominant clone, however, was at the center of the plot, whereas the vines that were very heavily infected in 2001 (vines 97, 98), where hardly touched by the pathogen in 2002. Each year, the focus of the disease turned out to be the site of the presence of the oospore that gave rise to the very infective clone. The 'predominance-of-one-clone' was the pattern of the epidemic development in both years.

The number of infection cycles during the 2001 epidemic was estimated to be six; one primary and five secondary (Table 4). At the first sampling, the predominant-2001 genotype appeared on many vines and also on a vine four rows away from the focus of the isolate, while the first rain events on May happened on the 6–10/05 ($Tav_{7-18\ May} = 15\ ^\circ C$, $RHav_{7-18\ May} = 85\%$, meaning 8–9 days incubation time). These facts suggest that the first primary infections occurred from 6/05 to 10/05. The primary lesions appeared from 15/05 to 19/05 and one secondary cycle of asexual reproduction had already taken place at the moment of our sampling. The second cycle of secondary infections occurred after a rain event on 26–27/05 and an increase of Tav to 23 °C (the incubation time was reduced to 4–5 days), the third after the rain on 7/06, the fourth occurred without the forcing power of a rain event, but probably because of the 3-h leaf wetness the night of the 20/06 and the last due to the very favorable conditions on 2/07 (16 mm of rain, 8-h leaf wetness).

The epidemic of 2002 started as two lesions that appeared on 16 May, after the rain events of 8/05 and 9/05 ($Tav_{9-16\ May} = 16.8\ ^\circ C$, $RHav_{9-16\ May} = 78\%$, corresponding to 7–8 days incubation time). The predominant-2002 genotype was not detected in the first sample; however, in the second sample it appeared with 43 lesions and that indicates that the genotype already existed in the date of the first sample, but we missed it either because it was still latent or because we had overlooked it. The third sampling was done on 13/06 after mild rainfalls from 22/05 to 26/05 and 6-h leaf wetness during the nights of 1/06 and 4/06. The Tav from the 23/05 to 13/06 was 21 °C, which is the optimum temperature for infections and corresponds to an incubation time of 4–5 days. This means that at least four secondary cycles may have taken place during that period and for that reason the disease was evenly dispersed over the entire plot. The last cycle was caused by the 5 h of leaf wetness on 25/06, the rain event on 27/06 and the increase of $Tav_{26\ June-2\ July}$ to 23.8 °C (the incubation time at this temperature is minimized to 4 days). In total, the epidemic was a result of seven infection cycles.

Two major common principles determined the epidemic development in both years. First, asexual reproduction was scarce and limited. The genotypes that reproduced themselves asexually in 2001 were 54.2% of the total 23 genotypes, while, in 2002, 19 clones out of the total 54 reproduced (35%). The majority of the genotypes did not spread further than a few vines around the vine of the first appearance of this clone. Second, one clone showed very high relative frequency and led the epidemic. Moreover, the predominant clones followed similar patterns and speed of dispersal; in both cases, the clones came (or almost came in the case of the predominant-2001) to the maximum possible distance they could reach inside the plot after two secondary cycles. The predominant-2001 was in 17–21 days (from the 15–19/05 – the dates that primary lesions appeared – until the 5/06, the date of the second sampling) 15.7 m far from the point of the oosporic

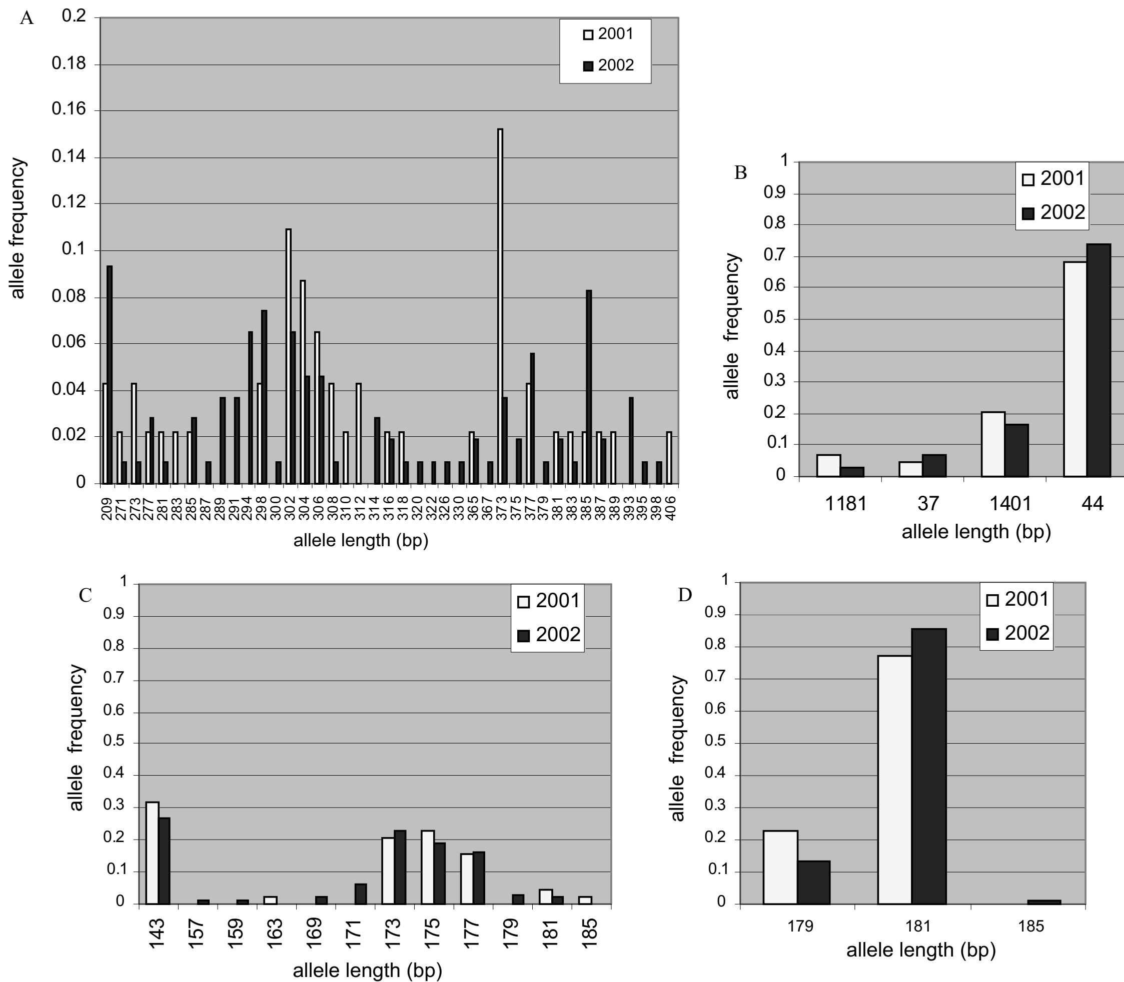

Figure 3. Allele frequency distributions of four microsatellite loci of *P. viticola* in the total populations of 2001 and 2002 after clone-correction; (A) locus GOB, (B) locus ISA, (C) locus CES, (D) locus BER.

Table 4. 2001 and 2002 climatic conditions conducive for *P. iticola* and number of infection events that were favored to occur*

Year	Rain (mm) (January–April)*	Rain (mm) (May–1st July)	Tav (°C) (May)	Tav (°C) (June)	RHav (%) (May)	RHav (%) (June)	No. of infection events
2001	167.2	60.2	17.8	22.7	80.2	59.8	6
2002	34	15	17.9	23.8	74.6	61.4	7

*Measurements were received from a meteorological station 500 m away from the plot.

infection of this genotype. The predominant-2002 was detected 12.6 m away from the focus in a time of 27–29 days (from the 15–17/05 – appearance of primary lesions – until the 13/06, the date of the third sampling), but we consider that the clone reached that distance much earlier because the climatic conditions favored many clonal cycles in between our second and third samplings.

Another interesting event common to both epidemics is the high appearance of new genotypes in every sample. In 2001, 41–71% of the genotypes of each sample were new ones, while, in 2002, the percentage was

even higher: 73–80% (Table 1). Even in very late samplings (e.g. the 2/07/2002), 30 out of the 41 genotypes detected, had not appeared in previous samples. Almost all these genotypes appeared with just a few lesions or even only once in the population and only some of them reproduced. The manner in which the majority of the new genotypes came out suggests that they were at the greater part primary infections and not migrating secondary sporangia, which would enter the plot in big numbers after large multiplication like the predominant clones of the samples we studied. This means that oosporic infections took place throughout the season, but most of them did not manage to cause secondary infections. Additionally, the persistency of genotypes through the samples is very limited: only three genotypes exist in more than three samples in 2001, whereas only the predominant-2002 persists in all the four samples of 2002. Three genotypes in each year appear in three samples, while 12 and 42 genotypes appear only in one sample, in 2001 and 2002, respectively.

Parallel to the appearance of new genotypes in each sample, a disappearance of old genotypes was observed. In 2001, three out of five clones from the first sample did not exist in the second, while one from the second sample did not appear in the third. In 2002, two out of five genotypes from the second sample did not exist in the third, and 11 out of 22 from the third were not present in the fourth. If all the lesions of each sample had been analyzed, the 'disappeared' genotypes would probably exist, but still their presence in the sample would have been really low. This situation is another consequence of the fact that asexual reproduction is a limited and outstanding event of a particular genotype.

The common pattern of the disease progress through the 2 years was caused by the existence, on both populations, of a genotype that managed to produce an exceptionally high number of copies. This observation led us to a genetic comparison of the two predominant genotypes. The predominant-2001 is 281-385/140-144/173-175/179-181, the predominant-2002 is 377-385/140-144/143-173/181-181 and it is observed that at each locus they share one – at least – of their alleles. The allele GOB-385 of the predominant-2002 is present only in one of the genotypes of 2001; in the predominant-2001. The allele GOB-377 is present in two genotypes; the 302-377/137-144/163-173/179-179 and the 308-377/118-140/143-143/181-181. Out of these two, though, only the second genotype has the allele CES-143, which is present in the predominant-2002 as well. This means that only two clones in the 2001 population could potentially be the parents of the predominant-2002. Additionally, the 308-377/118-140/143-143/181-181 clone was found on vine 50, very close to the site where the predominant-2002 first appeared (vines 48, 49). These facts lead us to the conclusion that the predominant-2002 can be a recombinant of 2001 population, which derived from the mating between the predominant-2001 and another clone of 2001 population in autumn 2001 on a vine in the middle of the plot.

Continuing the comparison between the predominant clones of 2001 and 2002, another observation is that both derive from early-matured oospores. The predominant-2001 clone came from one of the five first oosporic infections, while, the predominant-2002 derived from one of the six first oilspots. It could be argued that the predominant genotypes had more opportunity for asexual reproduction than genotypes derived from later oosporic infections. However, it is not reasonable why the rest of the oospores that produced primary lesions early did not also manage to cause many secondary infections. An explanation for the exceptional clonal reproducibility of the predominant genotypes could be a genetic advantage that was inherited from one clone to the other.

In order to estimate the ability of the predominant-2001 for sexual reproduction, we searched the 2002 population for other possible recombinants of the predominant-2001 with more individuals of 2001 population; only one more genotype was found – the 381-385/140-144/173-173/181-181. A probable second parent could have been the 381-387/144-144/173-173/179-181 clone. The 377-385/140-144/143-173/181-181 (the predominant-2002) and the 381-385/140-144/173-173/181-181 are the only clones produced by oospores-recombinants of the predominant-2001, which germinated and infected successfully 1 year after their birth. Eventually, the predominant-2001 was reproduced asexually to 242 clones and sexually to – at least – two oospores. The ability of the predominant clone for sexual reproduction appears to be lower than its ability for clonal reproduction, although the presence of oospores-recombinants in the soil should not be neglected.

Despite the common features the two epidemics show, the epidemic in 2002 was more serious than in 2001. Both the severity and the diversity of the disease in 2002 were higher. The magnitude difference between the two epidemics is negatively correlated to the rain precipitation of the 2 years. The precipitation from

January to April, which is correlated to the oospore maturation (Zahos, 1959), was five times more in year 2001 than in 2002 (Table 4). Also, the rain during the disease development (which triggers both primary and secondary infections) was four times more in 2001. Concretely, in May 2001, 22 nights were registered with leaf wetness with a total of 124 h, compared to May 2002 with 16 nights and a total of 74 h. The temperature did not differ between the two years, while the RH was much higher in May 2001. Our expectation was a harder disease at the more humid year; nevertheless the epidemic in 2002 was more severe than in 2001.

The disease progress in the plot can be partly explained by the given climatic conditions. On one hand, the spread of the predominant clones are in accordance with the rain events; the predominant-2002 did not multiply clonally as much as the predominant-2001 (Figure 4). On the other hand, in the population of 2002, there were three other genotypes apart from the predominant that caused secondary infections to more than eight vines, in contrast with 2001, where only the predominant was reproduced and spread to a great extent. In that case we regard the two of these clones (the b and c that appeared in the third sample) as migrants from neighbor vineyards that had similar abilities to multiply and infect like the predominant genotype. What cannot be explained by the environmental conditions is the higher amount of oosporic infections in 2002, while the conditions would not permit it. At this point, we should consider the importance of the epidemic of the previous year on the amount of inoculum that remains in the soil. A low or no disease in 2000 was maybe the reason for the low number of oosporic infections in 2001 and, consequently, for the limited epidemic. Respectively, the higher amount of inoculum in 2002 – produced by the 2001 epidemic – was one probable reason that the epidemic in 2002 was bigger.

In regard to an effective control strategy against the epidemics studied, we now address the elimination of the first lesions observed in the plot. According to rain events presented at the graphic (Figure 4), the first primary lesions (linear regressions of the curve of the predominant and of the total population) appeared on the 17–19 May in 2001 and on the 13–17 May in 2002,

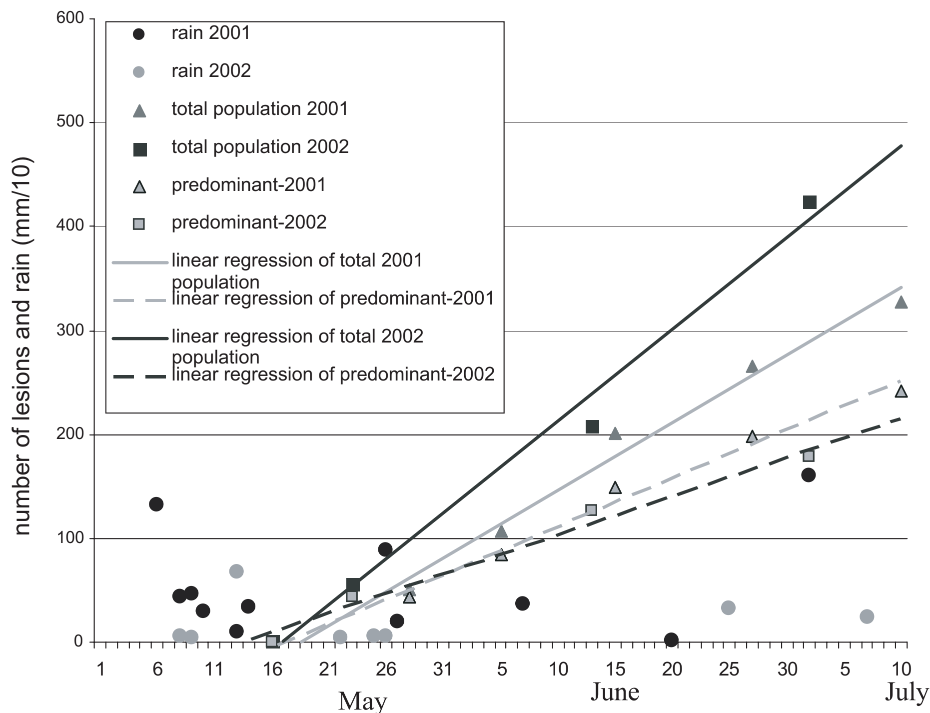

Figure 4. Progression of the disease caused by *P. viticola* and fraction attributed to predominant genotypes. Rain events and amount of precipitation are also indicated.

probably caused by the rain events of 6–10/05/2001 and 8–9/05/2002, respectively. A preventive treatment before the 6/05/2001 or just the cutting off of the infected leaves that appeared on the 15 infested vines on the 28/05/01 would have resulted in only 43 lesions at the end of the epidemic, which is 13% of the epidemic that occurred. The corresponding preventive actions in 2002 (treatment before the 8/05/02 or cutting off the infected leaves from the first 14 infested vines) would have led to a final population of 117 oilspots, which would be 27% of the real total population. Apparently, as far as the concrete plot of study is concerned and given the low average severity of the disease (2001: 3.3 lesions/vine, 2002: 4.3 lesions/vine), this alternative control measure could be really effective, although there is a difference in the success because of the difference in the genotypic diversity of the epidemics. The numerous oosporic infections until late in the season in combination with the migrants would enable the continued existence of the 2002 population.

The 'predominance-of-one clone' pattern of epidemic presented in the current paper could be compared with a similar situation observed in an Italian vineyard (Fiemme Valley, Trentino, Italy) (Gobbin et al., 2003b). In that case, a 330-vine isolated mountain plot was infested by 97 lesions of *P. viticola* from 23/6 to 14/7/00. Fifteen genotypes were identified but one of them covered 61% of the total population. The rain precipitation was low (31/5–20/7/00: 136 mm) and the vineyard was planted just the previous year. Conversely, there was observed another case in Blanquefort in France; a 280-vine plot in a plain area with often and long rains was infested with 557 lesions from 9/5 to 7/8/00 (Gobbin et al., 2003c) In this population 363 different genotypes were found and the most frequent one was only multiplied to 14 clones. The two main differences between these cases are the rain precipitation and the disease severity of the previous year(s). The rain is a factor that affects both primary and secondary infections; therefore, a severe and genotypically diverse epidemic occurred in the very humid region of Blanquefort, whereas in the dry sites of N. Aghialos and Tesero the epidemic did not grow large and only one genotype predominated. Similarly, the high severity of the prior epidemics in Blanquefort resulted in a very diverse epidemic; on the contrary, the low 2000 epidemic in N. Aghialos and the zero prior epidemic in Tesero produced limited disease.

After this analytical study of *P. viticola* population through a 2-year period, we obtained some evidence about the evolution of this population. The HW test showed that almost all the samples, from the very early ones – that derived mostly from the oospores that existed in the soil inside the plot – to the very late – where we expected migrants to have arrived – respected the HW principles. The only exception was the 2002d sample, where the null hypothesis was rejected at locus GOB. The very special peculiarity of this sample was the existence of six genotypes each of which shared most of its alleles with the predominant-2002 and differed from it only on one or two. After excluding these genotypes, the sample was in equilibrium. In that case, we can only speculate mutation having occurred during the high asexual multiplication of the predominant-2002 (it produced a total number of 178 copies), but at the moment we do not possess any proof for that. The result of the HW test for the rest of the samples, nevertheless, suggests that the particular *P. viticola* population is sexually reproduced under random mating and the evolutionary forces do not influence the equilibrium. However, it was previously shown that in 2002 migration occurred. From the way and speed of spread of the predominant genotypes (which showed the fastest dispersion), the gene flow seems to happen in short distance and the migrants belong probably to the same population, where the population of the studied plot belongs too. Moreover, the populations of the two years share less than half of their alleles at loci GOB and CES, while the 2002 population had a high number of private alleles at locus GOB. This means that there existed another source of genetic material for the 2002 population apart from the 2001 genotypes; this was older genotypes that remained for some years in the soil in the form of oospores. Finally, the population derives mainly from the gene pool in/on the soil which includes recombinants of the previous year (like the predominant-2002) and older genotypes-recombinants of years before the previous, whereas migrants are actually drawn from the broader gene pool, in which the gene pool of the certain 100-vine plot belongs as well.

Conclusion

The genetic screening of the two natural *P. viticola* epidemics proposes a reconsideration of our ideas about certain epidemiological aspects of the biology of the pathogen. Oosporic infections were observed throughout the season of our samplings and for at least 2 months, having a qualitative and quantitative impact

on the epidemic. The amount of oosporic infections appeared to be considerably affected by the prior epidemic and the quantity of primary inoculum potentially available in/on the soil. Under the given climatic conditions, the expansion force of the disease was not the secondary cycles that followed the primary infections, but the exceptional ability of a single genotype in each year to multiply and infect, dominating the population. The success of the secondary sporangia in causing infections, excluding the ones of the predominant clones, was regularly low and over short distances. If this way of sporangia spread is a general rule, then, we should expect low gene flow among the populations of the pathogen and, as a result, isolation by distance between the viticultural areas. At last, regarding the concepts under the Greek conditions, we detected, twice, that the epidemic started in the middle of the vineyard. This study represents a primary indication about the existence of new concepts in the epidemiology of *P. viticola* and further investigation under different conditions should take place in order to come to definite conclusions.

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